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Cap-Independent Translation of Poliovirus mRNA Is Conferred by Sequence Elements within the 5′ Noncoding Region

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Poliovirus polysomal RNA is naturally uncapped, and as such, its translation must bypass any 5′ cap-dependent ribosome recognition event. To elucidate the manner by which poliovirus mRNA is translated, we have determined the translational efficiencies of a series of deletion mutants within the 5′ noncoding region of the mRNA. We found striking differences in translatability among the altered mRNAs when assayed in mock-infected and poliovirus-infected HeLa cell extracts. The results identify a functional *cis*-acting element within the 5′ noncoding region of the poliovirus mRNA which enables it to translate in a cap-independent fashion. The major determinant of this element maps between nucleotides 320 and 631 of the 5′ end of the poliovirus mRNA. We also show that this region (320 to 631), when fused to a heterologous mRNA, can function in *cis* to render the mRNA cap independent in translation.

Poliovirus polysomal RNA, unlike most eucaryotic mRNAs, is not capped at its 5′ end, but rather terminates in pUp (15, 27). The mRNA contains a major translation initiation site approximately 750 nucleotides from the 5′ end (4). The long 5′ noncoding region of poliovirus mRNA (compared with an average of 100 nucleotides for eucaryotic mRNAs) is scattered with multiple upstream AUGs, of which only three are conserved in position among all three serotypes (17, 31, 39). These AUGs are not removed by splicing of the viral mRNA (4), and their function, if any, remains unclear. The first 650 nucleotides of the 5′ end are highly conserved (greater than 80% homology) among the three serotypes and are thought to be involved in some essential functions (39). Alterations within the 5′ noncoding region have been correlated with changes in neurovirulence, cytopathic effects, and translational efficiency (9, 19, 21, 28, 32, 38).

The mechanism by which poliovirus mRNA translates is enigmatic in several major respects. First, poliovirus RNA must bypass the requirement for a cap structure for its translation. This translation is efficient in extracts prepared from poliovirus-infected HeLa cells, in sharp contrast to the low translational levels exhibited by cellular mRNAs in these extracts. Second, it has been shown that poliovirus-mediated inactivation of the cap-binding protein complex (a translation initiation factor required for translation of most mRNAs; for a recent review, see reference 6), in conjunction with an as yet unidentified second event (2), allows poliovirus to usurp the cellular translational apparatus without the need to compete with cellular mRNAs (for reviews, see references 7 and 36).

A scanning model has been suggested to explain the manner by which eucaryotic mRNAs bind ribosomes (18). As postulated for other mRNAs, this model predicts that ribosomes bind at or near the 5′ end of poliovirus mRNA and proceed to scan the long leader region until the major translation initiation codon is reached. An alternative possibility is that ribosomes can bind internally near the major initiation site. In a recent report, Hassin et al. (13) presented evidence that such a mechanism occurs on the adenovirus type 2 mRNA of DNA polymerase. In a previous report, Herman (14) demonstrated internal initiation of translation on the vesicular stomatitis virus nonstructural mRNA, thus supporting the contention that internal initiation is possible on some eucaryotic mRNAs.

To gain a better understanding of the mechanism of translation initiation of poliovirus mRNA, we have analyzed the effects of deletions within the 5′ leader region of poliovirus mRNA on its translation in mock- and poliovirus-infected HeLa cell extracts. We show that poliovirus mRNA contains a *cis*-acting sequence within the 5′ noncoding region which mediates cap-independent translation. In addition, when fused to a heterologous mRNA species, this region confers upon the mRNA the ability to translate in a cap-independent fashion.

MATERIALS AND METHODS

Construction of deleted templates. Recombinant DNA techniques were performed by standard methods (24). The plasmid used to construct the deletion mutants was pP2-5′ (Fig. 1). This plasmid was constructed by inserting a *Hin*dIII-*Sma*I fragment of P2/Lansing cDNA (21) representing bases 1 to 1872 of the viral genome into pSP64. The 5′ 68 nucleotides of this cDNA fragment were derived from a P1/Mahoney cDNA (32) in which the first nucleotide of the viral RNA is preceded by 18 nonviral nucleotides, including a *Hin*dIII linker. Linearization of pP2-5′ with *Sma*I followed by in vitro transcription with SP6 RNA polymerase produces an RNA containing 24 nonviral nucleotides preceding the first poliovirus nucleotide.

The deleted templates Δ3′-631, Δ3′-381, Δ5′-733, and Δ5′-632 were constructed by cleaving pP2-5′ with combinations of restriction endonucleases *Hin*dIII, *Bal*I, *Eco*RV, and *Hae*III, removing the appropriate DNA fragments, and religating the ends. The deleted templates Δ5′-33, Δ5′-80, Δ5′-96, Δ5′-140, and Δ5′-320 were constructed by cleaving pP2-5′ with *Hin*dIII followed by treatment with nuclease

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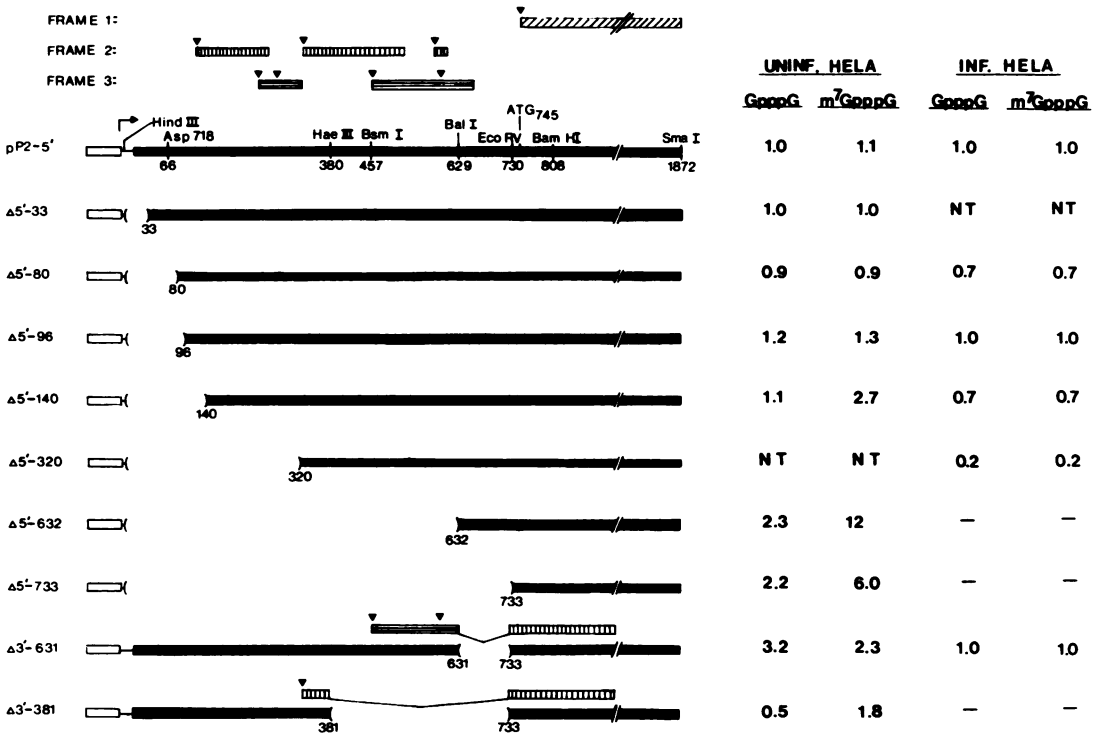

	UNINF. HELA		INF. HELA	
	GpppG	m^{7}GpppG	GpppG	m^{7}GpppG
pP2-5'	1.0	1.1	1.0	1.0
Δ5'-33	1.0	1.0	NT	NT
Δ5'-80	0.9	0.9	0.7	0.7
Δ5'-96	1.2	1.3	1.0	1.0
Δ5'-140	1.1	2.7	0.7	0.7
Δ5'-320	NT	NT	0.2	0.2
Δ5'-632	2.3	12	–	–
Δ5'-733	2.2	6.0	–	–
Δ3'-631	3.2	2.3	1.0	1.0
Δ3'-381	0.5	1.8	–	–

FIG. 1. Structure of deletion constructs of poliovirus RNA. ▭, SP6 polymerase promoter; ↱, transcription start site; ——, 24 extra, nonviral nucleotides between the SP6 promoter and the first nucleotide of the viral genome; ■, P2/Lansing viral sequences. RNAs transcribed from these templates contain the initiator ATG (shown at nucleotide 745) of the viral polyprotein encoding an ORF for a truncated poliovirus polyprotein consisting of VP4 (69 amino acids), VP2 (271 amino acids) and 37 amino acids of VP3 (predicted total molecular weight, 42,000 [21]). The ORFs defined by the AUGs in the 5′ noncoding region are represented at the top of the diagram; ▼, AUG positions. When a deletion causes an alteration within an upstream ORF resulting in termination within the major poliovirus polyprotein ORF, it is diagramatically represented above the respective construct. Deleted sequences are bracketed. The cleavage site of restriction enzymes used to produce the deletion mutants are shown on the pP2-5′ template. The numbers shown represent distances, in nucleotides, from the first nucleotide of the 5′ end of the viral template. The relative translational efficiencies of the deletion constructs obtained in mock- and poliovirus-infected HeLa extracts are indicated to the right. Radioactive bands corresponding to poliovirus protein product were quantified by soft-laser densitometry (LKB Instruments, Inc.), and the value obtained for capped unmethylated pP2-5′ mRNA was set as 1.0. NT; not tested; –, not detected.

BAL 31 for various periods. The truncated inserts were released from the vector by cleavage with *Sst*I and ligated into M13mp18 for sequencing by the chain termination technique (34). Deleted DNA templates were cleaved from the M13 vector by digestion with *Hin*dIII and *Sst*I and cloned into pT7SP6, so that transcription with SP6 polymerase yields positive-strand RNA transcripts.

Poliovirus/CAT fusion constructs were made as follows: Δ3′-70/CAT, Δ3′-461/CAT, Δ3′-631/CAT, and pP2/CAT were constructed by cleaving the parent poliovirus plasmid pP2-5′ with *Asp*-718, *Bsm*I, *Bal*I, and *Eco*RV, respectively. The resulting vectors were subjected to digestion with *Bam*HI (or *Eco*RI for Δ3′-461/CAT) and purified from a low-melting agarose gel as described previously (22). The chloramphenicol acetyltransferase (CAT) gene was isolated from pCAT (*Hin*dIII-*Bam*HI CAT fragment inserted between the *Hin*dIII-*Bam*HI sites of pSP64) by restriction with *Hin*dIII, blunt ending with Klenow, and excision with *Bam*HI, or by partial *Eco*RI restriction; it was then gel purified and directionally inserted into the various pP2-5′ deletion vectors with T4 DNA ligase.

The Δ5′-33/CAT, Δ5′-140/CAT, and Δ5′-320/CAT constructs were created by using the above strategy. Briefly, the corresponding parental vectors (Δ5′-33, Δ5′-140, and Δ5′-320, respectively) were restricted with *Eco*RV and *Bam*HI. The *Hin*dIII (blunt-ended)-*Bam*HI CAT gene was directionally inserted into the parental vectors with T4 DNA ligase. The *Bsm*I-*Eco*RV poliovirus fragment was inserted into pCAT. The fragment was rendered blunt ended with T4 DNA polymerase, ligated to synthetic *Hin*dIII linkers, restricted with *Hin*dIII, agarose gel purified, and ligated to *Hin*dIII-restricted pCAT.

Δ5′-320/Δ3′-461/CAT and Δ3′-461/CAT were constructed in a similar manner with Δ5′-320 and pP2-5′ as starting parental vectors. Δ5′-463/CAT was constructed from partial *Bsm*I restriction of pP2/CAT, blunt ending with T4 DNA polymerase, restriction with *Hin*dIII, and then filling in with Klenow. The resulting vector was religated with T4 DNA ligase. Dideoxy sequencing (34) was performed on Δ5′-463/CAT, Δ3′-461/CAT, and Δ5′-320/Δ3′-461/CAT to precisely map the nucleotide at which T4 DNA polymerase digestion terminated. Δ5′-465/Δ3′-631/CAT was constructed from Δ5′-465/CAT by digestion with *Bal*I and *Bam*HI. The CAT gene (*Hin*dIII-blunted *Bam*HI fragment) was then inserted into this vector with T4 DNA ligase. Δ5′-320/Δ3′-631/CAT was constructed in a similar fashion, except that Δ5′-320/CAT was used as the starting template.

In vitro transcriptions. Plasmids were linearized with *Sma*I (for pP2-5′ deletion constructs) or *Bam*HI (for pP2/CAT fusion constructs), and transcription reactions were carried

out by the method of Pelletier and Sonenberg (29), except that the GTP concentration was 50 μM. Yields of transcripts were calculated from the incorporation of [^{3}H]CTP into RNA. A portion of each transcription reaction was analyzed on 1.4% formaldehyde gels to ascertain that equal amounts of mRNA were used for translation. Autoradiography of the gels showed the presence of a single RNA species migrating at the appropriate size.

In vitro translations. Poliovirus type 1 (Mahoney strain) infection of HeLa cells was done as described by Lee and Sonenberg (23). Extracts from mock- or poliovirus-infected HeLa cells were prepared 3 h after infection, essentially as described previously (33), but with minor modifications (23). Extracts were judged to be infected by assaying for p220 proteolysis (a component of the cap-binding protein complex [8]), by the inability of oxidized reovirus mRNA to cross-link to cap-binding proteins (23), and by the detection of poliovirus proteins following in vitro translation of endogenous mRNA. Translation in HeLa extracts was carried out by the method of Rose et al. (33). Following translation of pP2-5′ deletion mRNAs, RNase T1 (200 U) and RNase A (1 μg) in 3 M urea–25 mM EDTA–0.5 mM phenylmethylsulfonyl fluoride was added and the incubation was continued at 30°C for 1 h. For gel analysis of translation products, portions from translation mixtures were mixed with sodium dodecyl sulfate sample buffer (20), boiled for 5 min, and applied on sodium dodecyl sulfate–10 to 15% polyacrylamide gels. Gels were fixed in 40% methanol–7.5% acetic acid, treated with En^{3}Hance (New England Nuclear Corp.), and exposed against X-ray film at −70°C. Translations for each experiment were performed at least twice with two different mRNA preparations.

RESULTS

To study the sequence requirements for the cap-independent translation of poliovirus mRNA, we used RNA transcribed from a type 2 poliovirus cDNA clone (30) with SP6 RNA polymerase. To avoid the analysis of several translation products generated by processing of the poliovirus precursor polyprotein (NCVP00), we used a truncated cDNA clone termed pP2-5′, extending to nucleotide 1872 in the VP3 region (Fig. 1). This cDNA template gave rise to the expected 1.8-kilobase RNA product following transcription in the SP6 system and analysis on formaldehyde gels (data not shown). Various deletion mutants in the 5′ noncoding region were constructed and are shown in Fig. 1. Following linearization of the plasmids, transcription by SP6 polymerase was performed in the presence of m^{7}GpppG or GpppG to generate methylated and unmethylated capped transcripts, respectively. It was previously shown that for cap-dependent mRNAs, the capped methylated (m^{7}GpppG...-terminated) form translates more efficiently than its capped unmethylated (GpppG...-terminated) counterpart (3). In addition, cap analogs (such as m^{7}GDP), but not their unmethylated counterparts, are specific inhibitors of methylated capped mRNA translation in vitro (16). We reasoned that deletions extending into the region responsible for cap-independent translation of the mRNA would render the mRNA cap stimulated. We attempted to identify the region responsible for cap-independent expression of poliovirus mRNA by comparing the translational efficiencies of the m^{7}GpppG...- and GpppG...-terminated mRNA species transcribed from the poliovirus deletion constructs. We have also translated uncapped poliovirus transcripts [terminating with a GMP-P(CH$_2$)P group] and found that they behave similarly to capped unmethylated transcripts (data not shown). All of these transcripts are more stable under translation conditions than pppG...-terminated mRNAs, which are rapidly degraded in several cell extracts (10).

Translation of pP2-5′ deletion mRNAs in extracts from mock-infected HeLa cells. We wanted to define features of the 5′ noncoding region of poliovirus mRNA that enable it to translate in extracts from poliovirus-infected cells, in contrast to most capped mRNAs, which do not translate in these extracts. The translation of mRNAs containing deletions within the 5′ noncoding region was first assessed by using extracts from mock-infected HeLa cells. The choice of this cell line was dictated by its almost universal use in studies of poliovirus. In this, and all subsequent in vitro translation experiments, *S*-adenosyl-L-homocysteine was included in the translation incubations to prevent methylation of unmethylated capped transcripts by endogenous methyltransferases (3). The results are shown in Fig. 2 and are summarized in Fig. 1. Translation of pP2-5′ mRNA in a HeLa mock-infected extract yields a 46-kilodalton (kDa) polypeptide (Fig. 2, lane 2). The predicted molecular mass of the VP4-VP2-VP3 polypeptide encoded by pP2-5′ mRNA is 42 kDa. Thus, the translation product migrates slightly slower than anticipated, but can be immunoprecipitated by anti-VP4 antibodies (data not shown). (The slower migration may be due to residual tRNA nucleotides remaining attached to protein following RNase treatment, which was performed after translation. The tRNA molecule could not be naturally released when ribosomes reached the end of the mRNA because of the lack of a termination codon.) Since VP4 contains only one methionine (derived from ATG_{745}), these results prove that translation on pP2-5′ commences with ATG_{745}. As anticipated, the translation of pP2-5′ mRNA was not stimulated by the presence of a methylated cap structure on the mRNA (Fig. 2, compare lane 3 with lane 2). The translational efficiency of deletion mutants Δ5′-33, Δ5′-80, and Δ5′-96 was similar to that of pP2-5′ mRNA and likewise not cap stimulated (lanes 4 to 9). A further deletion extending to nucleotide 140 resulted in a 2.4-fold difference in the translational efficiency between unmethylated (lane 10) and methylated (lane 11) capped mRNA. A significantly larger effect was obtained with deletion mutants Δ5′-632 and Δ5′-733 (lanes 12 to 15). The translation of capped Δ5′-632 mRNA is severalfold more efficient than that of pP2-5′ mRNA (compare lane 13 with lane 3); this is most probably due to the differences in the efficiencies between cap-dependent (Δ5′-632) and cap-independent (pP2-5′) mechanisms of initiation. It is of interest that synthesis of the major 46-kDa polypeptide from Δ5′-733 mRNA was greatly diminished, while ca. 27- and ca. 40-kDa protein species became more prominent (lanes 14 and 15). Polypeptides of these sizes are consistent with initiation at AUG codons that are present downstream of AUG_{745}. The short 5′ end of the Δ5′-733 transcript most probably does not allow for efficient recognition of the major AUG codon by 40S ribosomes. Translation of mRNA containing deletions upstream of nucleotide 733 shows that nucleotides 631 to 733 are not important for cap-independent translation (compare lane 16 with lane 17). In contrast, translation of methylated capped Δ3′-381 mRNA was 3.5-fold more efficient than its unmethylated counterpart (compare lane 19 with lane 18). The upstream overlapping open reading frames (ORFs) in Δ3′-631 and Δ3′-381 (shown diagramatically in Fig. 1) are not efficiently recognized by ribosomes, since the predicted polypeptides (ca. 17 kDa for Δ3′-631 and ca. 12 kDa for

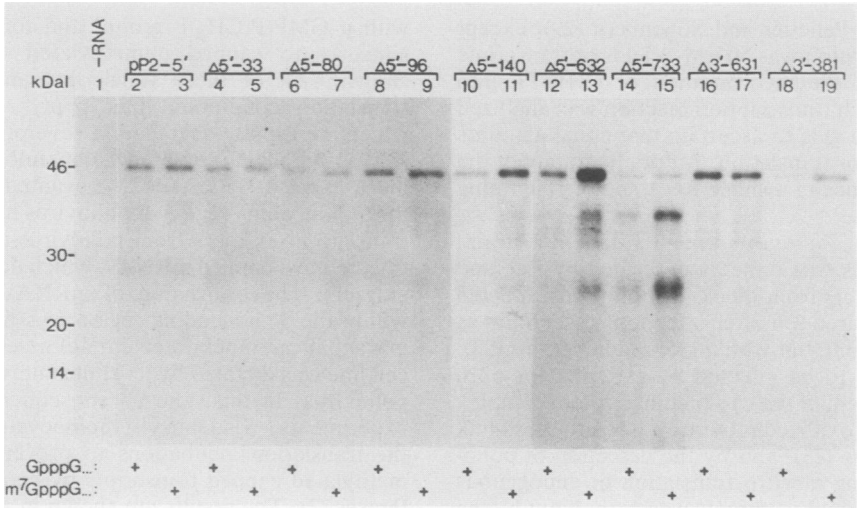

FIG. 2. Translation of pP2-5′ deletion mRNAs in mock-infected HeLa cell extracts. Translations were performed by using 12.5 μl as described in Materials and Methods, with 0.4 μg of pP2-5′ deletion mRNAs. Incubations were carried out for 60 min at 30°C followed by RNase treatment, and samples were processed for electrophoresis as described in Materials and Methods. Values obtained after scanning of the autoradiograph relative to the amount of protein synthesized from unmethylated capped pP2-5′ mRNA are indicated in Fig. 1. Kinetic analysis and dose-response curves indicated that the rates of translation were in the linear range. Lanes containing unmethylated (GpppG...) and methylated (m^7GpppG...) capped mRNAs are indicated.

Δ3′-381) were not detected. On the basis of the scanning model (18), one would expect the upstream ORFs to decrease translational efficiency, which definitely is not the case with Δ3′-631, in which even a slight stimulation is observed compared with pP2-5′ mRNA.

The absence of a translation termination codon on the pP2-5′ deletion mRNAs does not render termination rate limiting, since if this were the case, one would expect to see equal translational efficiency for all constructs. In addition, the ability to detect an effect of methylation of the cap structure on translation of several of the constructs (e.g., Δ5′-632; compare lane 13 with lane 12) argues that an event at the level of initiation is occurring. From the results shown in Fig. 2, we conclude that the major element responsible for cap-independent expression of pP2-5′ mRNA lies between nucleotides 140 and 631.

Translation of pP2-5′ deletion mRNAs in extracts from poliovirus-infected HeLa cells. The translation of mRNAs in poliovirus-infected HeLa cell extracts can occur only via a cap-independent mechanism (7, 36). pP2-5′ mRNA containing the sequence element(s) conferring cap-independent translation would therefore be expected to translate in extracts prepared from poliovirus-infected cells. Poliovirus pP2-5′ mRNA translated efficiently in an extract from poliovirus-infected HeLa cells, and translation was not dependent on a methylated cap structure (Fig. 3, lanes 2 and 3). Thus, the pP2-5′ mRNA contains all the information necessary for cap-independent translation. The major polypeptide synthesized had an apparent molecular mass of 42 kDa as compared with the 46-kDa polypeptide synthesized in extracts from mock-infected HeLa cells (compare Fig. 3 with Fig. 2). In addition, there is a minor product migrating above the major 42-kDa polypeptide product. It is likely that the translation product of pP2-5′ mRNA is processed by endogenous $3C^{pro}$ to yield VP4-VP2 (predicted molecular mass, 38 kDa) and a truncated VP3 product (4 kDa), which is not

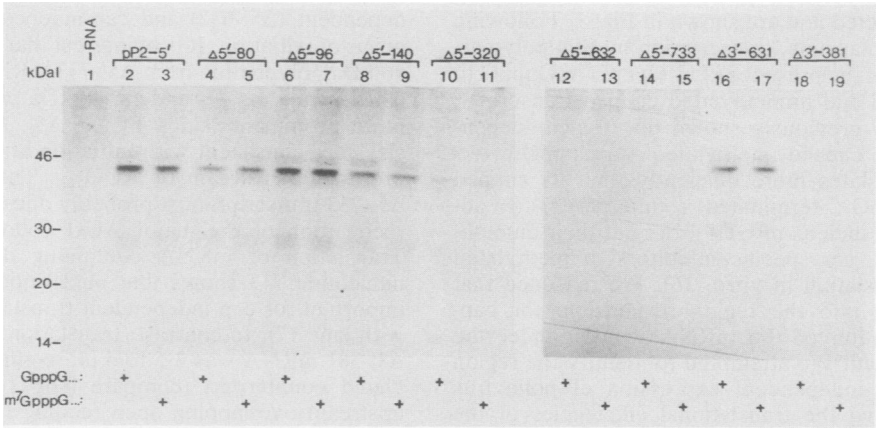

FIG. 3. Translation of pP2-5′ deletion mRNAs in poliovirus-infected HeLa extracts. Translations were performed as described in the legend to Fig. 2, and the products were resolved by polyacrylamide gel electrophoresis and visualized by fluorography. Lanes containing unmethylated (GpppG...) and methylated (m^7GpppG...) capped mRNAs are indicated.

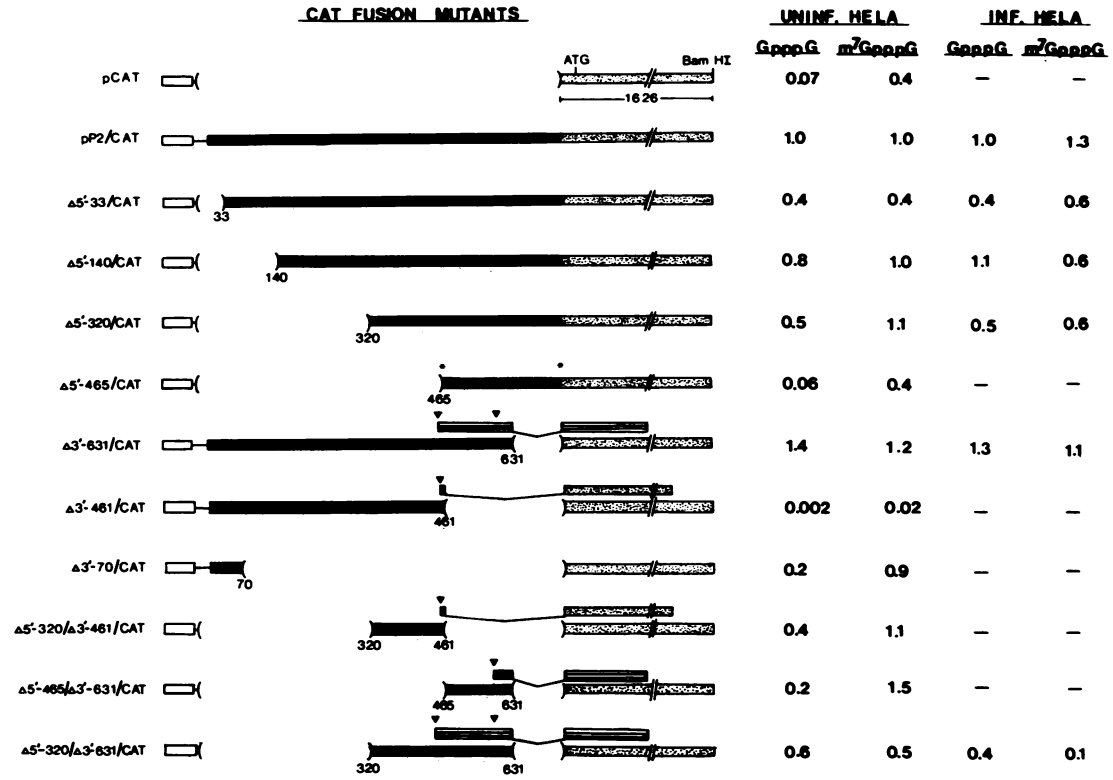

FIG. 4. Structure of fusion constructs of pP2-5′ and the CAT gene. ▭. CAT gene; ▬, sequences derived from pP2-5′. Alterations in ORFs caused by deletion mutagenesis are drawn above the appropriate construct. The asterisks above Δ5′-465/CAT denote the positions of synthetic *Hin*dIII linkers. The relative translational efficiencies obtained with the pP2/CAT fusion constructs are indicated to the right. The value obtained for capped unmethylated pP2/CAT was set as 1.0. –, Not detected.

seen. The residual 46-kDa protein is then unprocessed precursor. The translational efficiencies of mutants Δ5′-80, Δ5′-96, and Δ5′-140 were within 1.5-fold of that of pP2-5′ and were not cap stimulated (lanes 4 to 9). The translation of Δ5′-320 was reduced fivefold (lanes 10 and 11) compared with that of pP2-5′ (lanes 2 and 3), whereas expression of capped unmethylated and methylated mRNA from mutants Δ5′-632 and Δ5′-733 was undetectable (lanes 12 to 15). These results argue that sequences downstream of nucleotide 140 are important for cap-independent translation. The removal of 102 nucleotides just upstream of the major initiator AUG in Δ3′-631 did not affect cap-independent expression (compare lanes 16 and 17 with lanes 2 and 3), whereas removal of 354 nucleotides (Δ3′-381) resulted in an mRNA species incapable of translating in a poliovirus-infected extract (lanes 18 and 19). Taken together, the results shown in Fig. 2 and 3 position the major body of the element responsible for cap-independent translation between nucleotides 320 and 631, with contributions from sequences between 140 and 320. The fact that Δ5′-320 translates at a lower efficiency than pP2-5′ in poliovirus-infected extracts denotes a graded response to 5′-end deletion mutagenesis.

Translation of pP2/CAT deletion mRNAs in extracts from mock-infected HeLa cells. It is possible that the effects observed with the poliovirus mRNA 5′ noncoding sequences on translation are due to interactions between the 5′ noncoding and the downstream coding region. In addition, since none of our deletion constructs contained termination codons, the possibility (although very unlikely) exists that the rate of termination was somehow influencing (directly or indirectly) the expression from the various mutants. To experimentally address these potential pitfalls and determine whether the poliovirus mRNA 5′ noncoding region could function independently of the coding region, we fused the 5′ noncoding region of the deletion mutants to the bacterial CAT coding region. To better delineate the region responsible for cap-independent expression, additional constructs were produced and are shown in Fig. 4.

Translation of methylated and unmethylated SP6-derived CAT mRNAs was performed by using extracts from mock-infected HeLa cells in the presence and absence of m^7GDP to assess mRNA cap dependency. This was important to demonstrate, since CAT is a bacterial mRNA and its expression in a eucaryotic translation system may not a priori be cap dependent. Translation of unmethylated capped CAT mRNA yielded the expected sized polypeptide of 25 kDa (Fig. 5, lane 2). To demonstrate that the 25-kDa polypeptide corresponds to CAT, we immunoprecipitated the protein from the translation extract by using a monoclonal antibody directed against CAT (data not shown). The second polypeptide at ca. 16 kDa most probably arises from initiation at a downstream in-frame AUG codon that is expected to yield a polypeptide of this size (12). Translation of unmethylated CAT mRNA was not affected by the addition of m^7GDP (compare lane 3 with lane 2), whereas translation of methylated CAT mRNA was fivefold better than that of its unmethylated counterpart (compare lane 4 with lane 2). Addition of m^7GDP to methylated CAT mRNA reduced its translation to the level observed with the unmethylated CAT mRNA (compare lane 5 with lane 2). These results demon-

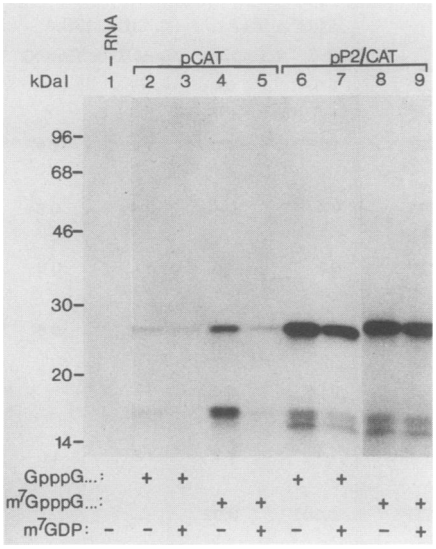

FIG. 5. Effect of cap analog on translation of mRNA derived from pCAT and pP2/CAT in mock-infected extract. Translations were performed with mRNA concentrations of 15 μg/ml. m^7GDP (0.1 mM) was added where indicated. Lanes containing unmethylated (GpppG...) and methylated (m^7GpppG...) capped mRNAs are indicated.

strate that translation of CAT mRNA in mock-infected HeLa cell extracts is cap stimulated. When the poliovirus mRNA 5′ untranslated region was fused to the CAT mRNA, translation of pP2/CAT transcripts was efficient and better than CAT mRNA, but not cap stimulated (compare lanes 8 and 6 with lanes 4 and 2, respectively). Translation of both methylated and unmethylated pP2/CAT mRNAs was not affected by the addition of m^7GDP (compare lanes 9 and 7 with lanes 8 and 6, respectively).

Figure 6A shows the effects of the deletions in the 5′ noncoding region of pP2/CAT on translational efficiency in mock-infected HeLa cell extracts. The translation of Δ5′-33/CAT (lanes 6 and 7) and Δ5′-140/CAT (lanes 8 and 9) was not stimulated by the presence of a cap structure, whereas that of CAT mRNA was (compare lane 3 with lane 2). These results indicate that nucleotides 1 to 140 are not essential for cap-independent translation of pP2/CAT. Deleting to nucleotide 320 and beyond, however, resulted in increasing cap-stimulated expression, as observed with Δ5′-320/CAT (2-fold, compare lane 11 with lane 10) and Δ5′-465/CAT (6.5 fold, compare lane 13 with lane 12). Fusing the CAT gene to the 5′-proximal 631 nucleotides of poliovirus mRNA (Δ3′-631/CAT) generated an mRNA which translated in a cap-independent fashion (lanes 14 and 15). When the 5′-proximal 461 nucleotides of the poliovirus genome were fused to the CAT gene (lane 16), there was a significant inhibition of CAT mRNA translation (0.2% as compared with pP2/CAT; lane 4) and translation was cap stimulated (10-fold, compare lane 17 with lane 16). The cap dependency of Δ3′-461/CAT was further confirmed by m^7GDP inhibition studies (data not shown). The reasons for the low translational level obtained with this deletion construct are addressed in the Discussion. When the deletion mutant Δ3′-70/CAT was tested, translation was efficient and cap stimulated (lanes 18 and 19). Taken together, these results place the major cap-independent region between nucleotides 320 and 631, with some effect of nucleotides 140 to 320. To substantiate this conclusion, we created three subclones containing fragments spanning the majority of this region (see Fig. 4 for constructs). Translation of Δ5′-320/Δ3′-461/CAT (Fig. 6A, lanes 20 and 21) or Δ5′-465/Δ3′-631/CAT (Fig. 6B, lanes 3 and 4) was cap stimulated, although the degree of dependency was different between the two constructs. Only Δ5′-320/Δ3′-631/CAT (Fig. 6B, lanes 5 and 6) translated in a cap-independent fashion. Therefore, nucleotides 320 to 631 can function in this assay, albeit at a lower efficiency, consistent with this region containing the major determinant(s) for cap-independent translation.

Translation of pP2/CAT deletion mRNAs in extracts from poliovirus-infected HeLa cells. The conclusions drawn from the studies of translation of pP2/CAT mRNAs in extracts

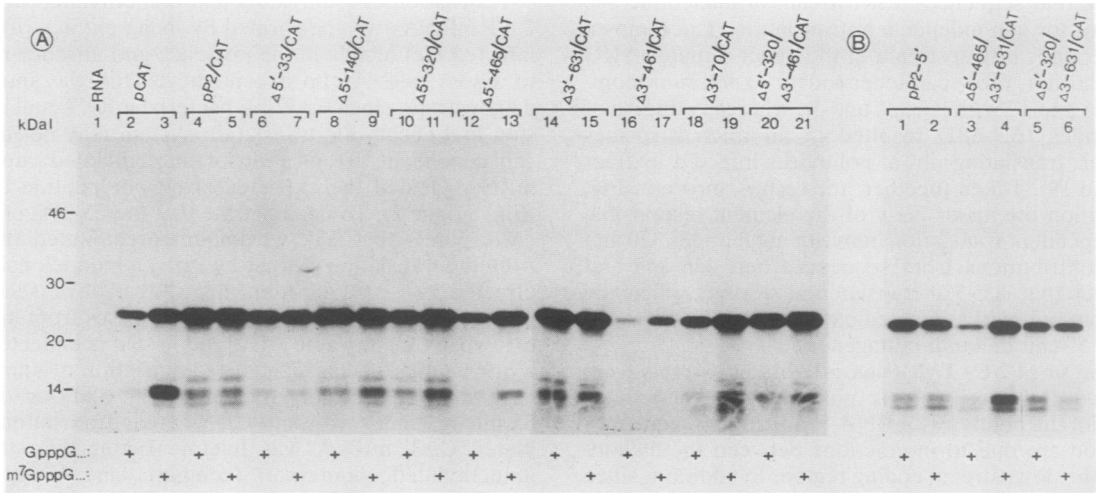

FIG. 6. Translation of pP2/CAT fusion mRNAs in extracts from mock-infected HeLa cells. Translations were performed with mRNA concentrations of 30 μg/ml and processed as described in Materials and Methods. Lanes containing unmethylated (GpppG...) and methylated (m^7GpppG...) capped mRNAs are indicated.

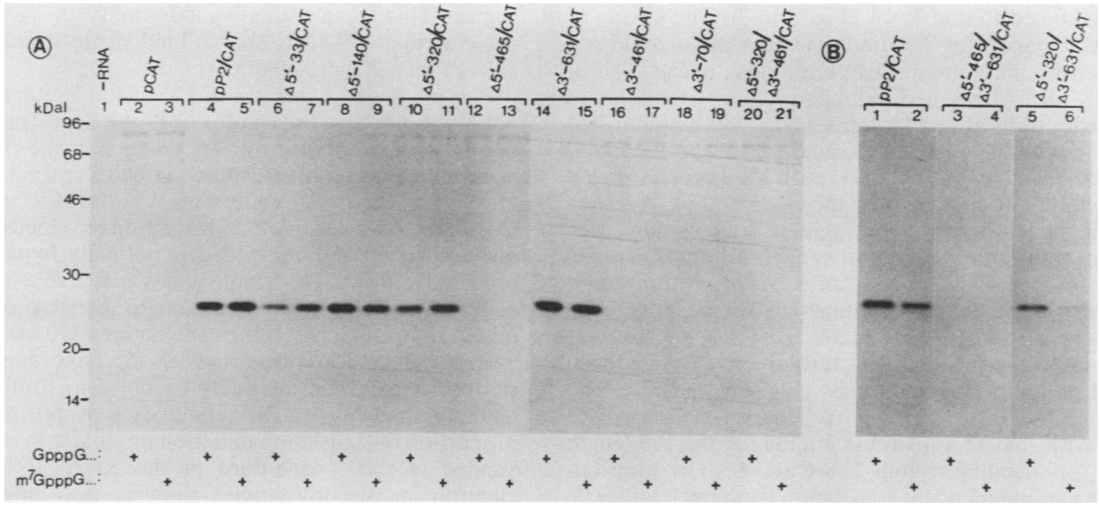

FIG. 7. Translation of pP2/CAT deletion mRNAs in extracts from poliovirus-infected HeLa cells. Translations were performed with mRNA concentrations of 30 μg/ml and processed as described in Materials and Methods. Lanes containing unmethylated (GpppG...) and methylated (m^7GpppG...) capped mRNA are indicated.

from mock-infected cells were further supported by results obtained from translation in extracts from poliovirus-infected cells (Fig. 7). Translation of unmethylated or methylated CAT mRNA in extracts from poliovirus-infected cells was undetectable, as anticipated (Fig. 7A, lanes 2 and 3). Fusion of the poliovirus 5′ noncoding sequences to CAT mRNA resulted in synthesis of the CAT product (lanes 4 and 5). Deletion mutant Δ5′-33/CAT mRNA translation was reduced twofold as compared with that of pP2/CAT mRNA (lanes 6 and 7), and Δ5′-140/CAT (lanes 8 and 9) translated with similar efficiency to pP2/CAT. The expression of Δ5′-320/CAT (lanes 10 and 11) was reduced twofold compared with that of pP2/CAT. Removal of nucleotides beyond 320, however, yielded an mRNA species (Δ5′-465/CAT) not capable of translating in a poliovirus-infected HeLa cell extract (lanes 12 and 13). The 5′-proximal 631 nucleotides of poliovirus conferred the ability to express CAT in a poliovirus-infected HeLa cell extract as assessed by translating Δ3′-631/CAT (lanes 14 and 15), but further 3′ deletions, Δ3′-461/CAT (lanes 16 and 17) and Δ3′-70/CAT (lanes 18 and 19), abolished translation. The fusion mutants containing internal 5′ poliovirus sequences, Δ5′-320/Δ3′-461/CAT (lanes 20 and 21) and Δ5′-465/Δ3′-631/CAT (Fig. 7B, lanes 3 and 4), also did not translate in the poliovirus-infected extract. Only Δ5′-320/Δ3′-631/CAT, containing nucleotides 320 to 631 from the poliovirus genome, translated in a cap-independent fashion (Fig. 7B, lanes 5 and 6). (The reason for the lower translational efficiencies of methylated capped Δ5′-320/Δ3′-631/CAT [Fig. 7B, lane 6] compared with its unmethylated counterpart [lane 5] is not clear.) These results are consistent with those obtained with the pP2-5′ mutants (Fig. 2 and 3), with some differences involving nucleotides 140 to 320; the possible reasons for this are addressed in the Discussion.

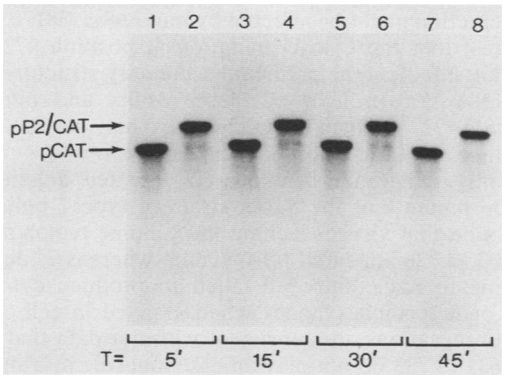

FIG. 8. Stability of pCAT and pP2/CAT mRNAs in poliovirus-infected HeLa cell extracts. ^{32}P-labeled mRNA (30 ng; specific activity = 1.5×10^7 cpm/μg) was incubated in a translation system prepared from a poliovirus-infected HeLa extract as described in Materials and Methods. At the various times indicated, a portion was removed, diluted 10-fold in proteinase K buffer (0.1 M Tris hydrochloride [pH 7.5], 12 mM EDTA, 0.15 M NaCl, 1% sodium dodecyl sulfate) and incubated with proteinase K (400 μg/ml) at 30°C for 30 min. Samples were phenol extracted, ethanol precipitated, and analyzed on a 1% formaldehyde–agarose gel. The gel was treated with En^{3}Hance, dried, and exposed against XAR-5 film (Eastman Kodak Co.). The mRNA species and times of incubation are indicated.

We confirmed in all of the experiments described above that the stability of the GpppG...-terminated mRNAs was identical to their m^7GpppG...-terminated counterparts. The stabilities of CAT and pP2/CAT mRNAs at different periods of translation in a poliovirus-infected extract were assayed (Fig. 8). When analyzed on a 1.0% formaldehyde agarose gel, the two transcripts were found to have similar stabilities (Fig. 8). Thus, the observed translational results are not due to differential mRNA turnover rates.

DISCUSSION

The results in this paper demonstrate the existence of a *cis*-acting element(s) within the poliovirus mRNA 5′ noncoding region necessary for its translation under conditions that prevent cap-dependent translation. This element also confers cap-independent translation to a heterologous RNA

(CAT) when fused to that RNA (Fig. 5 to 7). The effects of the mRNA 5′ noncoding deletions on translation could not be explained by differential mRNA stability, because all of the deleted mRNAs studied exhibited similar stabilities in the different translation systems (Fig. 8; unpublished results). These findings are not peculiar for the CAT mRNA, since similar results were obtained with the herpes simplex virus thymidine kinase gene (unpublished results). We have mapped the major body of the element that confers cap-independent translation to poliovirus mRNA between nucleotides 320 and 631. This conclusion is based on translation data obtained with deletion mutants of the 5′ noncoding region, and particularly Δ5′-320/Δ3′-631/CAT mRNA that translates in a cap-independent fashion in extracts from mock- and poliovirus-infected cells (Fig. 6B and 7B). The efficiency of the latter mRNA is reduced only twofold compared with that of constructs containing the full-length poliovirus 5′ noncoding region. There may also be contributions to the cap-independent translation from nucleotides 96 to 320. For example, deletion of nucleotides 1 to 140 (Δ5′-140) produced an mRNA that was slightly (2.4-fold) cap stimulated in uninfected extracts (Fig. 2) and translated slightly less efficiently in infected extracts (Fig. 3). However, when the same deletion was introduced into pP2/CAT, there was little if any cap stimulation and no loss of translation efficiency in infected extracts (Fig. 7). Deletion of nucleotides 1 to 320 in pP2-5′ (Δ5′-320) produced an mRNA with fivefold-reduced expression in a poliovirus-infected extract (Fig. 3, lanes 10 and 11). However, when the same deletion was introduced into pP2/CAT, only a twofold reduction in translation efficiency in infected extracts was observed (Fig. 3). The reasons for these differences in translation are not clear, although it is possible that translation is influenced, to some degree, by the coding region of the pP2-5′ mRNA. In any case, it is apparent that the element(s) specifying cap-independent translation in mock-infected extracts allows translation of these mRNAs in poliovirus-infected HeLa extracts.

What might be the mechanism which enables poliovirus mRNA to initiate translation via a cap-independent fashion? Our data show that a large area is encompassed by the cap-independent region (nucleotides 320 to 631) and that a graded translational response (as opposed to an all-or-none effect) is obtained in poliovirus-infected extracts upon deletion of nucleotides 140 to 320 (Fig. 3, compare lanes 8 and 9 with lanes 10 and 11). One possibility is that an internal guide sequence in the 5′ noncoding region of poliovirus mRNA acts to direct ribosomes to the mRNA, bypassing the requirement for a cap structure. Another possibility is that a region lacking secondary structure is required for internal binding. mRNAs with little potential to form stable secondary structure at their 5′ ends were shown to function in extracts from poliovirus-infected HeLa cells (37). For example, the naturally capped viral alfalfa mosaic virus type 4 mRNA, which contains little secondary structure at its 5′ end (11), is able to translate in poliovirus-infected HeLa cells (37). Thus, it is possible that poliovirus mRNA contains a single-stranded region enabling 40S ribosomes to bind internally. We and others have presented data to support a model by which a cap-binding protein complex (eIF-4F) binds to the 5′ cap structure of mRNA, followed by the binding of eIF-4A and eIF-4B, with subsequent unwinding of mRNA secondary structure to facilitate ribosome binding (36). It is conceivable that eIF-4A and eIF-4B bind directly to the *cis*-acting element(s) within the 5′ noncoding sequence of poliovirus mRNA, followed by internal binding of ribosomes. This is an attractive possibility in light of results showing that eIF-4A is able to bind single-stranded RNA in an ATP-dependent fashion (1).

It is of interest that the CAT fusion mutant containing poliovirus nucleotides 1 to 461 (Δ3′-461/CAT) translates very poorly compared with mRNA containing the full-length 5′ noncoding region in HeLa mock-infected extracts (Fig. 5 and 6). This effect is not seen with Δ3′-70/CAT, implying the existence of a translational restrictive element between nucleotides 70 and 461 which is normally bypassed on the pP2/CAT mRNA. This element may play a role in preventing 5′-end-mediated initiation that could interfere with internal initiation occurring between nucleotides 320 and 631.

Experiments by Dorner et al. (5) have suggested that ribosomes can bind internally on poliovirus mRNA. Those authors showed that in reticulocyte lysate a significant proportion of translation initiation originates in the P3 region located in the 3′ one-third of the RNA. Evidence from electron microscopy studies showing ribosome binding to the mRNA P3 region supports this conclusion (25). Recent experiments by Shih et al. (35) using cDNA hybridization to arrest translation of encephalomyocarditis virus mRNA demonstrated that the region near nucleotide 450 but not the 5′-proximal 338 nucleotides is required for translation. This raises the possibility that internal initiation is also possible on encephalomyocarditis virus mRNA, although direct proof is still lacking.

A single base change (C to U at base 472) in the 5′ noncoding region of the poliovirus genome was found to be a major determinant of the attenuation of the Sabin type 3 polio vaccine (9, 40). A single base change is thought to contribute to the attenuation of the Sabin type 1 strain as well (26). The molecular mechanism by which this mutation alters poliovirus neurovirulence is not understood. However, it has been found that mRNAs from attenuated strains of poliovirus types 1 and 3 exhibit diminished translational activity in extracts from Krebs-2 ascites cells compared with mRNAs from their virulent counterparts (38). Consequently, it was suggested that nucleotide sequences encompassing position 472 might interact with translation factors and that this interaction might be affected by mutations (38). It is also of interest that the C-to-U mutation at position 472 has a significant effect on the predicted secondary structure of the viral RNA (9). In light of these results and our data, nucleotide 472 (and flanking nucleotides) may play an important role in the translation of poliovirus RNA.

Recently, Kuge and Nomoto (19) created deletion and insertion mutants of the Sabin strain of type 1 poliovirus. They found that viruses lacking the genome region at positions 600 to 726 appeared fully viable, whereas 3′ deletions extending to nucleotide 580 failed to produce cytopathic effects or detectable plaques when assayed in cell culture. These observations are consistent with our data that nucleotides 631 to 733 do not play an essential role in translation of poliovirus mRNA. We are currently performing experiments in vivo to demonstrate the relevance of our in vitro studies to the in vivo translation of poliovirus. In this regard, isolation of viable mutants which have lost the ability to translate in a cap-independent manner would be helpful.

In summary, our results identify a *cis*-acting element within the 5′ noncoding region of poliovirus RNA. The major element maps between nucleotides 320 and 631 and enables poliovirus mRNA to translate in a cap-independent fashion. The identification of *trans*-acting factors which mediate this effect will be of importance in elucidating the mechanism by which poliovirus mRNA is translated.

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